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A Method of Analysis for the Individual Tetracyclines of a Mixture in Biological Fluids

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For the evaluation of a therapeutically useful combination of three tetracycline antibiotics (tetracycline, chlortetracycline and demethylchlortetracycline), a method of analysis capable of estimating the concentrations of the individual components of the mixture in biological fluids was desired. A fluorometric procedure, sensitive to concentrations of 0.1 to 0.2 mg/l of each antibiotic in the presence of the other two was developed.
In the procedure, chlortetracycline is determined by conversion to the fluorescent isochlortetracycline. Tetracycline is then determined on the same aliquot by conversion to anhydrotetracycline and measurement of the fluorescence of an aluminum complex of this derivative. Demethylchlortetracycline is determined by difference after application of a method which measures total tetracyclines.

Für die Entwicklung einer therapeutisch wirkungsvollen Kombination von drei Tetrazyklinen war es erforderlich eine analytische Methodik auszuarbeiten, die erlaubt, Konzentrationen der einzelnen Komponenten in biologischem Material zu bestimmen. Es wurde ein fluorometrisches Verfahren entwickelt, dessen Empfindlichkeit ausreicht, um Konzentrationen von 0,1—0,2 mg/l eines jeden Antibiotikums in Gegenwart der beiden anderen Komponenten zu bestimmen.
Chlortetrazyklin wird als das fluoreszierende Isochlortetrazyklin bestimmt. Das gleiche Material kann zur Tetrazyklinanalyse benutzt werden, das als Anhydrotetrazyklin-Aluminiumkomplex fluorometrisch bestimmt wird. Die Menge von Dimethylchlortetrazyklin ergibt sich nach Abzug dieser beiden Werte von der Gesamtmenge.

The fluorescence properties of the tetracycline antibiotics have been used for the study of these compounds since their introduction (1, 2). Numerous procedures based on fluorescence have been devised for the quantitative determination of these antibiotics in biological samples in order to study their pharmacokinetic properties (3—5).
Recently, in these laboratories, an evaluation of combination therapy using three of these antibiotics in a single dose form has been under investigation. The three tetracyclines are tetracycline hydrochloride, chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride. For reasons which will be described elsewhere, these have been combined in a ratio of 1:1:0.6 to form a "triple tetracycline". To aid in the evaluation, a chemical method of analysis for each of the individual tetracyclines in the presence of the other two in biological fluids was desired as a valuable adjunct to the commonly employed microbiological analysis. The latter procedure does not have the capacity to make such a distinction. The proper sequential use of the various procedures available provided such an analytical method.

Materials and Methods

Reagents

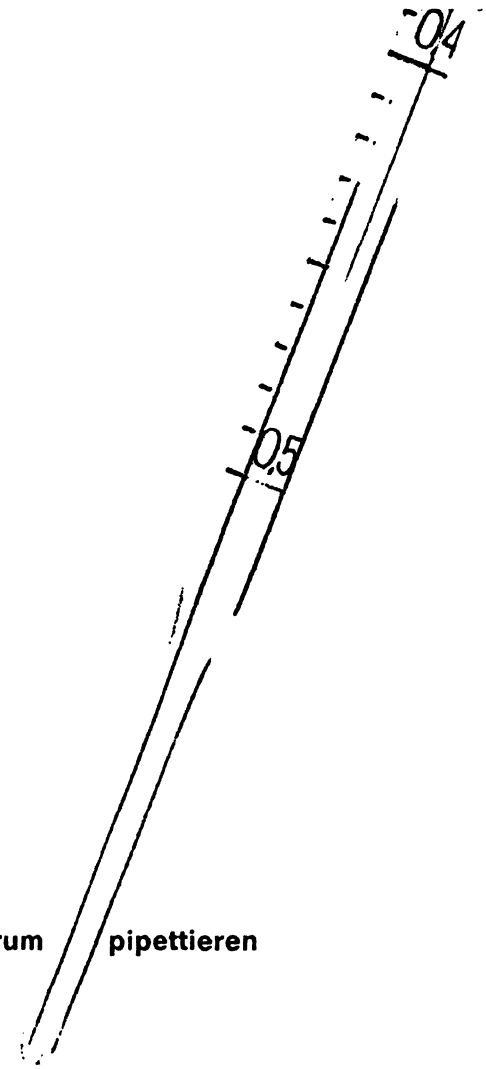
All aqueous solutions were prepared from distilled water made metal-free by distillation in glass apparatus.
0.16M calcium chloride in 1.8N trichloroacetic acid
0.9M sodium barbital
30% trichloroacetic acid
0.1M disodium ethylenediaminetetraacetic acid in 2N sodium hydroxide
6N hydrochloric acid
6N sodium hydroxide
1M citrate buffer, pH 4.6 (prepared by mixing 1M citric acid and 1M trisodium citrate until the desired pH is attained).
0.1% aluminum chloride ($\cdot 6H_2O$) in absolute ethanol (several hours of mixing at ambient temperature is required to effect this solution).
Reagent grade ethyl acetate
Reagent grade chloroform.

Procedure

1. To a 1.0 ml sample of serum as well as a reagent blank and 1.0 ml volumes of 1 mg/l standard solutions of tetracycline (TC), chlortetracycline (CTC) and demethylchlortetracycline (DMCT)[1]) add 4.5 ml of water followed by 1.0 ml of 0.16M calcium chloride in 1.8N trichloroacetic acid.
2. Shake, centrifuge and transfer a 4.0 ml aliquot to a clean glass stoppered centrifuge tube.
3. Add 3.0 ml ethyl acetate and 4.0 ml 0.9M sodium barbital. Shake for 5 minutes.
4. Centrifuge and read the fluorescence of the ethyl acetate phase in a spectrophotofluorometer[2]) using an activating wavelength of 400 nm and a fluorescence wavelength of 520 nm.
5. To a second series of 1.0 ml volumes of sample, reagent blank and 1 mg/l standards add 8 ml of water and 1.0 ml of 30% trichloroacetic acid.
6. Shake, centrifuge and transfer 8.0 ml of the supernatant to a clean tube.
7. Add 1.0 ml of 0.1M disodium ethylenediaminetetraacetic acid in 2N sodium hydroxide. Mix.
8. Permit to stand 30 minutes and read fluorescence in the spectrophotofluorometer, activating the fluorescence at 350 nm and reading at 420 nm.
9. Transfer an 8.0 ml aliquot of the same solution to a clean glass stoppered centrifuge tube and add 1.0 ml of 6N hydrochloric acid.
10. Heat for 1 hour in a water bath at 50° C.
11. Cool and add 1.0 ml of 6N sodium hydroxide and 1.0 ml of 1M citrate buffer, pH 4.6.
12. Add 2.0 ml of chloroform and extract. The pH of the upper phase solution should be checked at this point. It should be

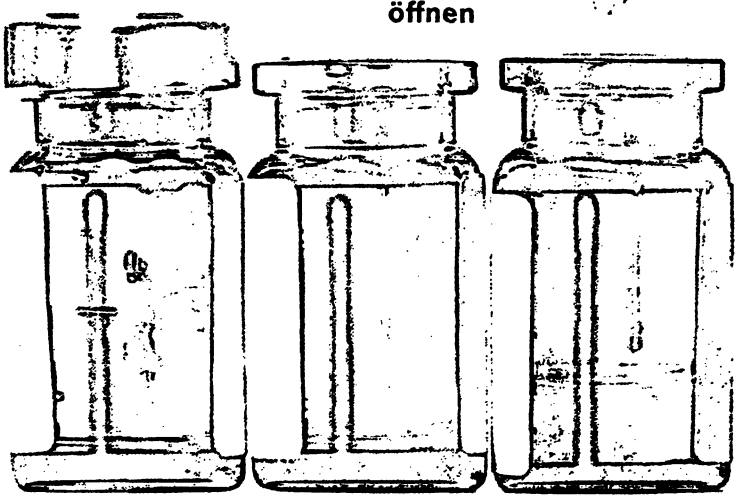
[1]) *Abbreviations:* TC = tetracycline, CTC = chlortetracycline, DMTC = demethylchlortetracycline.
[2]) An Aminco-Bowman Spectrophotofluorometer was used for the work reported here. A Turner filter fluorometer, fitted with appropriate interference filters for fluorescence activation, has been used successfully for the analysis.

Reagenziensätze für Analysen in einem Glas mit Asal-Monopacks



Serum pipettieren

öffnen



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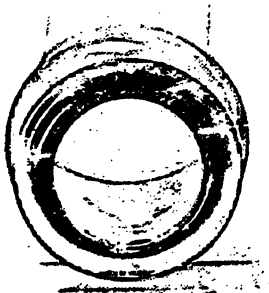

GOT-Monopack
Transaminaseaktivität
im Serum

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between 4.0 and 5.0 and, if it is not, it should be adjusted to within these limits with either sodium hydroxide or hydrochloric acid and the extraction repeated.
13. To a 1.0 m/ aliquot of the chloroform extract, add 1.0 m/ of 0.1% aluminum chloride in absolute ethanol.
14. Permit to stand at least 1 hour and determine the fluorescence of the solution when activated at 475 nm and read at 550 nm.

Calculations

Chlortetracycline and tetracycline are determined directly from the comparison of the fluorescences read at step 8 and at step 14 of Procedure with those found on the standard solutions of these compounds.
A factor (F) for each of the three compounds is determined from the readings made on the 1 mg/l standards at step 4 of Procedure. This is done by multiplying the reading of the photometer by the meter multiplier setting and subtracting the blank value similarly determined. An F value for the sample minus blank is also determined (F_S).
The concentration of demethylchlortetracycline is determined by substituting in the following formula:

$$C_{DMCT} = \frac{F_S - F_{CTC} \cdot C_{CTC} - F_{TC} \cdot C_{TC}}{F_{DMCT}}$$

where C = concentration of the antibiotic in the sample.

Results und Discussion

For the analysis of the individual components of the triple tetracycline mixture, advantage is taken of differences shown by each component in reactivity toward acids and bases (Fig. 1). Under mildly alkaline conditions, chlortetracycline has been shown to convert to isochlortetracycline (6), a highly fluorescent derivative (7). Neither tetracycline nor demethylchlortetracycline undergoes this reaction using the conditions described, presumably because the chemical structure of these compounds does not display the steric condition of bulky groups in *peri* position (carbons 6 and 7) as does chlortetracycline (8). This difference in response to alkalinity has been used as the basis of a differential spectral analysis of individual components in a tetracycline-chlortetracycline mixture (9).
All three of these compounds undergo dehydration in acid solution to form an anhydro derivative (6). However, demethylchlortetracycline is more resistant to acid degradation than either tetracycline or chlortetracycline (10). Isochlortetracycline cannot be converted to anhydrochlortetracycline so that a mixture of the three antibiotics can be submitted to a sequence of reactions such that chlortetracycline is converted to isochlortetracycline and tetracycline subsequently converted to anhydrotetracycline, leaving demethylchlortetracycline in its original state. Both isochlortetracycline and anhydrotetracycline can be determined by fluorometric procedures (7, 11) in which there is no interference to the assay of one by the other or by demethylchlortetracycline. An estimate of the latter compound can be made by the subtraction of the results for chlortetracycline and tetracycline from the analysis of total tetracyclines done on a separate aliquot of the original sample by a third fluorometric procedure to which all three of the antibiotics respond (3).

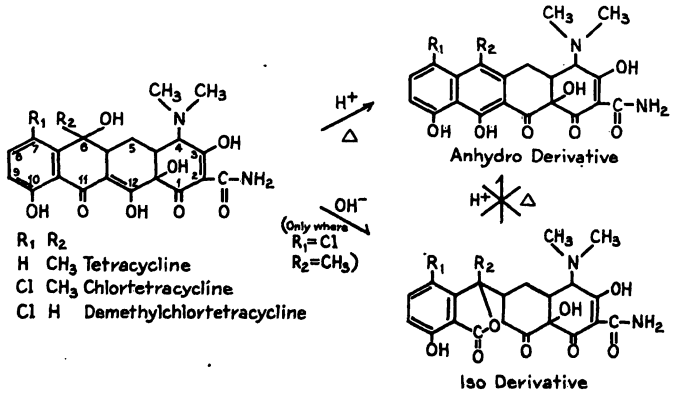

Fig. 1. Structures of Tetracycline Antibiotics and Their Derivatives

The conversion of chlortetracycline to isochlortetracycline takes place readily at room temperature under the influence of alkali. The reaction is somewhat inhibited by the presence of divalent metal ions so ethylenediaminetetraacetic acid is added to the system along with the alkali to prevent the interaction of the metal ions with the antibiotic. By testing a number of chlortetracycline solutions under the conditions described in the Procedure it was found that complete conversion was effected in 30 minutes. Isochlortetracycline in an alkaline solution gives a fluorescence which is activated at 350 nm and read at 420 nm. The limit of sensitivity of the assay for isochlortetracycline and therefore for chlortetracycline in plasma is of the order of 0.1 mg/l.
The conversion of tetracycline to anhydrotetracycline is carried out in an approximately 0.6N acid solution at 50° C. The temperature and acidity of the solution were determined by testing several variations of conditions until one was found which quantitatively carried out the desired reaction without dehydrating the demethylchlortetracycline. Following the formation of the anhydrotetracycline, it is extracted into chloroform and the fluorescent aluminium chelate used for its measurement. Anhydrotetracycline-aluminum fluorescence is activated at 475 nm and read at 550 nm.
The determination of demethylchlortetracycline is carried out through the use of the method described by KOHN (3). All three of the antibiotics respond to this procedure. However, calculations are somewhat complicated by the fact that the fluorescence response of each is different. In general the relative responses are of the order of 9:8:3 for demethylchlortetracycline, tetracycline and chlortetracycline, respectively. It can be seen from these figures that an error involved in the determination of tetracycline will have a much greater effect on the eventual assignment of a value for demethylchlortetracycline than will a corresponding error in the determination of chlortetracycline. In one respect this is unfortunate since the most probable cause of error in the overall assay procedure involves the conversion of tetracycline to anhydrotetracycline wherein there is the possibility that demethylchlortetracycline might also be converted to an anhydro derivative.
On the other hand, the response ratio can be used to advantage when the assay results are compared to those obtained on the same sample by the microbiological procedure (12). The responses of the three

Tab. 1
Fluorometric Analysis of Individual Tetracyclines in Various Mixtures and Comparison of the Calculated Tetracycline Equivalents with Microbiological Assay

Sample	Added — mg/l				Found — mg/l				
	TC	CTC	DMCT	Total in TC Equiv.	TC	CTC	DMCT	Total in TC Equiv.	Total in TC Equivalents Microbiological Assay
1	0.2	0.3	0.5	3.05	0.24	0.28	0.43	2.79	3.15
2	0.1	0.1	0.4	1.75	0.10	0.08	0.37	1.57	1.64
3	0.5	0.2	0.3	2.30	0.55	0.21	0.23	2.19	2.25
4	1.0	0	0	1.00	1.10	0	0	1.10	1.02
5	0	0.8	0.2	4.20	0.01	0.77	0.16	3.96	4.47
6	0	0	1.0	3.00	0.03	0	0.95	2.88	2.95

antibiotics to this procedure are of the order of 4.5:3:1 for chlortetracycline, demethylchlortetracycline and tetracycline, respectively. This ratio has led to the use of "tetracycline equivalents" in the expression of the microbiological activity of an antibiotic sample where a unit concentration of chlortetracycline is equal to approximately 4.5 equivalents of tetracycline and a unit concentration of demethylchlortetracycline is equal to approximately 3 equivalents of tetracycline. Where these factors are applied to the results obtained by the fluorometric procedure to express the total antibiotic present in terms of tetracycline equivalents, it is difficult to conceive of a combination which would adhere to the fluorometric ratio and correspond to the microbiological assay without this combination actually being present in the sample. Thus the assay procedure is provided with an excellent check on its accuracy if correspondence between microbiological and fluorometric tetracycline equivalents can be achieved.

Table I shows the results obtained on a series of aqueous solutions of the three tetracycline compounds in various combinations. These results indicate a reasonable correspondence between the fluorometric and microbiological assays even where the concentrations of the individual components are 0.1 to 0.2 mg/l. However a slight bias toward higher tetracycline values at the expense of demethylchlortetracycline values can be seen in the values obtained fluorometrically. This is due either to the conversion of a small amount of the demethylchlortetracycline to the anhydro compound in the acid-heat degradation step for tetracycline or to the extraction of a fraction of the unchanged demethylchlortetracycline into chloroform and subsequent dehydration of this compound in chloroform solution under the influence of aluminum chloride. At this time, the magnitude of this bias is not known. For the present it would appear that the bias is not sufficiently large to invalidate the use of the assay procedure.

Tab. 2
Analyses of Individual Tetracyclines in Serum of Dogs Which Received 25 mg/kg of "Triple Tetracyclines"

Animal Number	Hour	TC mg/l	CTC mg/l	DMCT mg/l	Calculated TC Equivalents	Microbioligical TC Equivalents
1	0	0	0	0	0	0
	2	0	0	.02	.06	0
	4	0	0	.02	.06	0
	7	.06	.08	0		
	24	.06	.08	0	.42	.26
2	0	.01	0	0	.01	0
	2	1.18	.85	.45	6.35	6.50
	4	.88	.69	.46	5.37	5.20
	7	.59	.53	.12	3.34	3.20
	24	.16	.12	.03	.79	.90
3	0	0	.02	0	.09	0
	2	.24	.16	.12	1.32	1.38
	4	.21	.09	.16	1.10	1.15
	7	.19	.23	0	1.23	.90
	24	.07	.06	0	.34	.20
4	0	0	.03	0	.14	0
	2	.74	.62	.29	4.40	4.80
	4	.56	.45	.28	3.43	4.00
	7	.41	.41	.07	2.47	2.40
	24	.08	.06	0	.35	.38
5	0	.01	0	0	.01	0
	2	.24	.38	.09	2.22	2.70
	4	.16	.23	.03	1.29	1.30
	7	.11	.22	0	1.10	.80
	24	.09	.13	0	.68	.65

Table II shows the results obtained on a series of serum samples taken various hours after a single oral dose of 25 mg/kg "triple tetracyclines". The tetracycline mixture was in the ratio of 10:10:6, chlortetracycline: tetracycline: demethylchlortetracycline. A close correspondence between microbiological and fluorometric "tetracycline equivalents" is achieved indicating the validity of these results as described above.

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